SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS DETECTION OF NITRATE AND NITRITE

Dejan Baskić[1], Ivan Jovanović[1], Petar Ristić[2], Vladimir Jakovljević[3], Đorđije Delibašić[3] and Nebojša Arsenijević[1]
[1]Department of Microbiology and Immunology,[2]Public Health Institute, Kragujevac, [3]Institute of Physiology, Faculty of Medicine, University of Kragujevac, Serbia and Montenegro

SPEKTROFOTOMETRIJSKA METODA ZA ISTOVREMENU DETEKCIJU NITRATA I NITRITA

Dejan Baskić[1], Ivan Jovanović[1], Petar Ristić[2], Vladimir Jakovljević[3], Đorđije Delibašić[3] i Nebojša Arsenijević[1]
[1]Katedra za mikrobiologiju i imunologiju, [2]Institut za zaštitu zdravlja, Kragujevac, [3]Katedra za fiziologiju, Medicinski fakultet, Univerzitet u Kragujevcu, Srbija i Crna Gora

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SAŽETAK

Koncentracija azot monoksida može se meriti brojnim metodama, ali kratak poluživot i niske izmerene vrednosti azot monoksida umanjuju im praktični značaj, te ostaju nepodesne za kliničku primenu. Nedostaci pomenutih metoda mogu se eliminisati merenjem stabilnih metabolita NO, kao što su nitriti i nitrati. U ovom radu opisali smo modifikaciju metode koja se zasniva na redukciji NO_3 i simultanoj detekciji svih krajnjih produkata oksidacije NO. Nivo nitrita i nitrata je određivan u serumu dodrovoljnih, zdravih davalaca. Serum je pre izvođenja testa deproteinizovan.. Redukcija nitrata u nitrite postignuta je vanadijumom(III). Za merenje koncentracije nitrita korišćena je kolorimetrijska detekcija sa Griess-ovim reagensom. Koncentracija nitrata izračunavana je kao razlika koncentracije ukupnih NO_x, određene u prisusvu VCl_3 i Griess-ovog reagensa, i koncentracije NO_2, merene samo u prisustvu Griess-a. Na osnovu dobijenih rezultata, zaključili smo da je metoda osetljiva do 0,5μM NO_3 i može se primenjivati na brojnim tečnostima uključujući serum, plazmu i supernatant kulture ćelija. Merni opseg metode značajno je veći pri upotrebi HCl u Griess-ovom reagensu, u odnosu na H_3PO_4, Sa druge strane, bez obzira na sastav Griess-ovog reagensa (HCl ili H_3PO_4), merni opseg metode je širi kada se reagensi inkubiraju na 37°C.

Ključne reči: azot monoksid, nitrati, nitriti, vanadijum(III), Griess-ov reagens.

ABSTRACT

Concentration of nitric oxide can be measured by variety of methods. Its short half life and values of low detectability decrease clinical importance of these methods. Deficiencies of methods used for NO measurement can be eliminated by measurement of stable NO products such as nitrites and nitrates. In this study we have modified a method for simultaneous evaluation of nitrate and nitrite concentrations.

Human sera were collected from blood donor volunteers. Before testing the samples were deproteinized. Reduction of nitrate was achieved with vanadium(III). Nitrite concentration was measured by Griess reaction. The nitrate concentration was calculated as difference of NOx (nitrites and nitrates), determinated in presence of vanadium(III) and nitrites concentration. This assay has shown sensitivity to 0,5μM NO_3 and is useful in variety of fluids including serum, plasma and cell culture media.

We have examined the influence of various factors on detection of NOX, such as reagent composition, volume, and temperature. The method has shown higher sensitivity when Griess reagent with HCl was used, compared to use of Griess reagent with H_3PO_4. It has been also noticed that regardless of which Griess reagent composition was used, sensitivity of this reaction was higher when samples were incubated at 37°C instead at 25°C. All incubations lasted 30 minutes.

Key words: nitric oxide, nitrate, nitrite, vanadium(III), Griess reagent.

INTRODUCTION

Nitric oxide (NO) is a short-life mediator of numerous physiological processes from neurotransmission, muscle relaxation and vasodilatation to antipathogenic and tumoricidal responses (1–5). Biosynthesis of NO from L-arginine by participation of oxygen is only possible in cells that provides NOS activity (Nitric Oxide Synthase) (6). There are two NO synthase enzymes: constitutive cNOS, in endothelial cells, which produces small quantity of NO without induction and inducible iNOS, which on stimulation synthesizes large amount of NO (7). Overproduction of NO can be a promoter of variety of diseases (8–9). That is the main reason for development of reliable techniques for detecting NO production.

Although NO concentration can be measured by many methods (chromatography, electron paramagnetic resonance, electrochemistry) (10), the short half-life and low concentrations of NO (11) reduce the practical significance of these tests, making these procedures unsuitable for clinical use as well as for scientific purposes. Deficiency of mentioned methods can be eliminated by measuring the stable NO metabolites, in particular nitrite (NO_2) and nitrate (NO_3).

Nitrites (NO_2) are representing the stable, final product of the oxidation of NO in aqueous solution (12). Nitrates (NO_3) are formed by reaction of NO with oxyhemoglobin or superoxide (reaction of oxidation) (13,14). In addition, nitrites are converted to nitrates by oxyhemoglobin (12–15). Consequently, plasma, serum and urine, as mediums with oxyhemoglobin and superoxide, predominately contain nitrates, while significant nitrites can accumulate in non-heme-containing fluids such as cerebrospinal (16).

The simplest and most frequently applied method for detection of nitrite anions employs colorimetric detection with Griess reagent, reagent that makes purple azo-colors with nitrites.

Since the conventional Griess reaction has limitations regarding sensitivity (1–5 mM) (17) and inability to detect NO_3 (which doesn't undergo diazotisation), several modifications have been adapted. The detection limit of the assay can be enhanced (linear to 0.2 μM NO_2) (18) by substitution of dapsone for sulfanilamide. Additionally, the total concentration of oxidative endproduct of NO can be determined by converting (reduction) NO_3 to NO_2.

Correspondence:
Dejan Baskic, PhD
Department of Immunology and Microbiology
Faculty of Medicine, Universty of Kragujevac
Svetozara Markovica 69, 34000 Kragujevac
Phone: +38134335224, Fax: +38134331345
e-mail: dejan.baskic@gmail.com

Reduction of NO_3 to NO_2 is usually achieved with reducing metals such as cadmium (19) or enzymatically through use of bacterial NO_3 reductase. NO_3 reduction by cadmium involves handling of a toxic metal. Moreover, cadmium is a relatively nonspecific reducing agent and shows unsatisfactory activity at low concentrations of NO_3 (18). Reliability and safety favor the use of nitrate reductase over cadmium. The sensitivity of this assay extends to the low range, under 1μM, which is generally sufficient for measurement of NO_2 and NO_3 in serum, plasma and urine as well as in iNOS-containing cell culture media. A disadvantage of this method is interference of NADPH with the Griess reaction, what can be a reason of measurement failures (20).

In this assay we described the modified method (Miranda et al. 2001) that combines reduction of NO_3 and measurement of NO2 in a single step. Reduction is achieved with vanadium(III), which has a reduced toxicity in comparison with cadmium and does not require removing before measurement of NO_2 . Vanadium(III) is routinely used to reduce NO_3 to NO at temperatures exceeding 80°C for chemiluminescent detection (21).

However, at reduced temperatures, NO_3 reduction is finishing by NO_2 formation. Adding the Griess reagents eliminates requirement for chemiluminescent detecting, but also enables convenient simultaneous detection of NO_2 and NO_3.

MATHERIAL AND METHODS

Serum Preparation

Human sera, collected from blood donor volunteers, were stored at -70°C until use. The serum was deproteinized before testing. Protein precipitation was achieved by two methods:

Protein precipitation by alcohol. In 1500μl tubes was added 100μl of plasma and 900μl of mixture methanol-diethyl ether (3v/1v). Extracts were incubated on room temperature over night, and than centrifugated five minutes at 13000rpm (Eppendorf 5415D, Eppendorf, Germany). Supernatant was transfered to another tube and freezed on -20°C until use. Just before testing extracts were defreezed and centrifugated two minutes at 15000 rpm with aim to get more lucid sample.

Protein precipitation by acid solution. In 1500μl tubes was added 100μl of 3M perchloric acid, 400μl of 20M EDTA and 200μl of serum. Extracts were incubated in ice 20 minutes folowed by occasional mixing and than centrifugated at 13000rpm for five minutes. To supernatants, transfered into another tubes, was added 120μl 2M kalium-carbonate to neutralize extracts. The neutralized extracts were stored at -20°C until testing. Just before use they were defrost and centrifugated in order to reduce potassium-perchlorate particules presence.

Nitrate and nitrite analysis

Experiments were performed at room temperature (25°C) and at 37°C. Nitrate standard solution was serially diluted (200–1.6μM, final concentration after adding other reagenses 67–0.5μM) in a 96-well, flat-bottomed, polystyrene microtiter plate (MTP), in final volume of 100μl. The diluting medium (destilated aqua) was used as the standard blank. After loading the plate with serum samples (100 μl), addition of VCl3 (100 μl) to each well was rapidly followed by adding of fresh Griess reagent (100μl). Reduction of nitrate to nitrite require acidic enviroment. Because of this the detecting solution (Griess), with low pH value, was applied during reduction. Presence of Griess reagent prevented loss of signal wich could be caused by diffusion of NO from the solution. Sample blank consisted of diluting medium and Griess reagent. Nitrites were measured on similar way as nitrates. Nitrites measurement in comparizon to nitrates detection differ in adding of VCl_3. Actually, samples and nitrite standards were only exposed to Griess reagents. In either case the absorbance was measured at 540 nm (Multiplate Reader 230S, Organon) following 30 min. incubation. Concentrations of NO_X (NO_3 + NO_2) and NO_2 in samples were determined using Xia software for data analising, based on standard curvature, which was got by linear regression of absorbance values for each standard reduced for blank values. It is therefore allowed to determine the nitrite and total NO_x concentrations for a particular sample in the same MTP so that the conditions are identical for each measurement. These values were then subtracted to give the nitrate concentration.

Chemicals

N(1-Naphthyl) ethylene-diamine dihydrochloride (NEDA), sulfanilamide (SULF), vanadium(III) chloride (VCl_3), (Sigma, Germany), hydrochloric acid (HCl), phosphoric acid (H_3PO_4), methanol, diethyl ether, perchloric acid ($HClO_4$), EDTA, potassium-carbonate (K_2CO_3), (Zorka-Sabac, SCG).

Stock Solutions

Saturated solution of VCl_3 (400 mg) was prepared in 1 M HCl (50 ml). Excess solid was removed with a nylon-66 syringe filter, and the blue solution was stored in the dark at 4°C for less than two weeks. Development of a lighter blue color indicated oxidation, after which the solution was discarded.

Griess-reagent was prepared *ex tempore*, just before the experiment by mixing equal amounts of stocks: 2% (w/v) sulfanil-amide, dissolved in 5% HCl or H_3PO_4 and 0,1% (w/v) aqueous solution of N-(1-naphthyl) ethylene-diamine, dihydrochloride (NEDA). Complete dissolution of SULF required stirring and heating. Solution was left through night on room temperature, after which solution was filtered to remove trace particules. Both solutions were stable for several months when stored in the dark at 4°C and were discarded if colored.

Nitrite and nitrate solutions in H_2O (10mM) were prepared fresh daily.

RESULTS AND DISCUSSION

Assay sensitivity and dependence on temperature and reagent composition. Standard curves of serially diluted nitrate concentrations are represented in Fig. 1. Extinctions represented NO_3 concentations from 0.5 to 67μM (after triple dilution of start concentrations).

The assay sensitivity was increased by substitution of HCl for H_3PO_4 in the Griess reagents followed by incubation either at room temperature (25°C) either at 37°C. Namely, extinction range in presence of HCl was from 0.161–1.240 while in presence of H_3PO_4 was significantly lower (0.149–0.326) (Figure 1). On the other hand, sensitivity of method was higher at 37°C incubation irrespectivelly on Griess-reagent composition (HCl, or H_3PO_4). In presence HCl extinctions of standards with the highest concentraton were significantly higher at 37°C than at room temperature incubation (1.240 vs 0.556). Differences were observed even when H3PO4 was used instead of HCl (0.326 vs 0.258) (Figure 2). All MTP were incubated for 30 minutes.

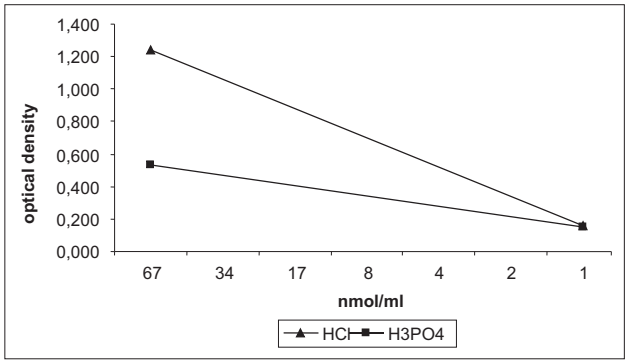

Figure 1. NO_3 reduction and NO_X detection by VCl_3/ Griess method. NO_3 standards (0.5–200μM) afther 30 min. incubation at 37°C, using Griess reagent containing 5% HCl, respectively 5% H_3PO_4.

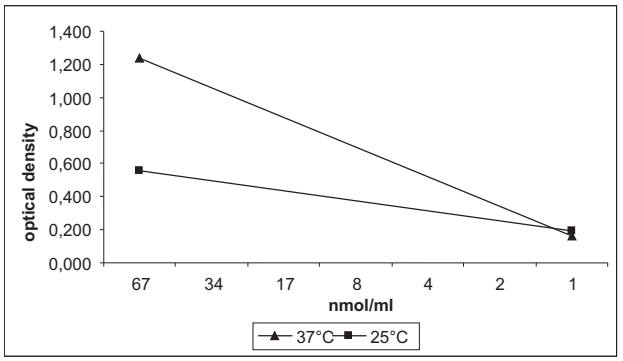

Figure 2. NO_3 reduction and NO_X detection by VCl_3/ Griess method. NO_3 standards (0.5–200μM) afther 30 min. incubation at 25°C, using Griess reagent containing 5% HCl, respectively 5% H_3PO_4.

Influence of varied reagent volume. Equivalent volumes of standard/sample, reducing agent and Griess reagents (300 ml total), effectively reduce the NO_X concentrations by threefold. The intensity of the detection could be modified by altering the volume of the assay solutions as shown in 3 and 4 figures. Although linearity was achieved under all conditions examined (variance of either VCl3 or Griess 20–100 μl), higher volumes of the VCl3/Griess are corresponded to higher absorbance values (Fig. 3 and 4).

Time dependence. In the presence of nitrite, the measured intensity rapidly reached a maximum (at 37°C). Reduction of nitrate by vanadium(III) proceeded at a much slower rate. That makes period between 30 and 45 min optimal for NOX detecting, at 37°C.

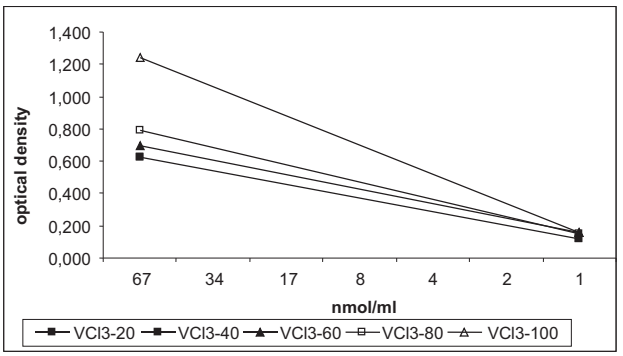

Figure 3. Effect of varied volume of VCl_3 on reduction and measurement of NO_3 standards after 30 min incubation at 37°C, using 5% HCl Griess reagent (100 μl

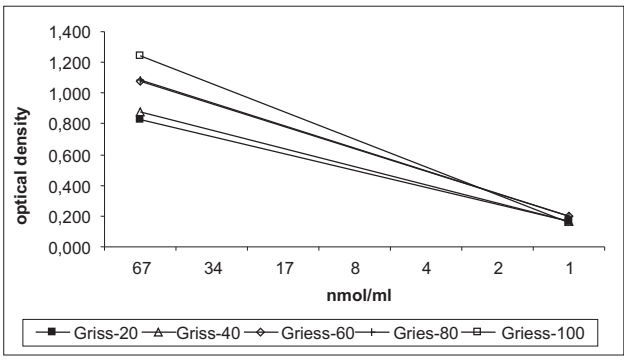

Figure 4. Effect of varied volume of 5% HCl Griess reagent on reduction and measurement of NO_3 standards, after 30 min incubation at 37°C (100 μl standard + 100μl VCl_3+ 20–100μl G(HCl)).

For expected concentrations of total NOX lower then 25 μM, incubation times should not exceed 30 minutes because longer incubations could cause appearance of artifacts.

Clinical use. Biological samples contain nitrates primarly, while nitrites can accumulate in cell cultures expressing NOS. Nitrates concentration calculated as difference between NO_X, measured in presence of VCl_3 and Griess reagent, and NO_2 concentration, measured in presence of Griess reagent. Efficiency of NO_3 reduction into NO_2 is important for of the precision method. Comparizing of NO_3 and NO_2 standard extinctions with an appropriate concentrations we concluded that converting efficiency was about 95%.

To confirm the validity of the assay, the measured levels of NOX in normal human sera were directly compared to those obtained by standard method with Griess only. Analysis by VCl_3/Griess resulted in values from 1 to 20 μM NO_3. The results obtained with Griess only were from 0 to 4 μM NO_2 . Both methods were conducted under identical conditions.

CONCLUSIONS

We have reported a new modified method for simultaneous detection of NO_3 and NO_2 concentrations that involves reduction of NO_3 by vanadium(III) and detection of total values of endproducts of NO oxidation. A range of sensitivity from 0.5 μM to >1 M NO_3 can easily be accommodated by modifications in reagent composition, volume, temperature and incubation time.

The assay does not require specialized equipment and is suitable for large-number analysis of minimal sample volumes. Comparison with other assays demonstrates the sensitivity, rapidity, simplicity, accurency and reliability of this convenient method for simultaneous measurement of NO_3 and NO_2 in biological samples.

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